VIROLOGY 206, 724–730 (1995)

Site-Directed Mutagenesis Confirms the Involvement of Carboxylate Groups in the Disassembly of Tobacco Mosaic Virus

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Received August 9, 1994; accepted October 7, 1994

Electrostatic repulsion between carboxylate groups across subunit interfaces has for many years been recognized as important in the disassembly of simple plant viruses. In the coat protein of tobacco mosaic virus (TMV), the amino acids Glu50 and Asp77 have been proposed as examples of such carboxylate groups. Site-directed mutagenesis has been used to replace these amino acids by Gln and Asn, respectively. Increased virion stability, together with reduced infectivity and reduced capacity for long-distance transport within the host plant confirms that the negative charges on the side chains of these amino acids are involved in the disassembly of TMV. Mixing purified mutant coat proteins with wild-type virions under appropriate conditions stabilizes the virions to alkaline disassembly and reduces their infectivity. It is suggested that transgenic plants expressing such mutant coat proteins could have enhanced resistance to virus infection.

Carboxylate groups have been found at subunit interfaces in the structures of many plant viruses (*1*), often forming calcium binding sites. Examples include the spherical viruses tomato bushy stunt virus, southern bean mosaic virus, and satellite tobacco necrosis virus, and the rod-shaped tobacco mosaic virus (TMV). Interactions between carboxylate groups provide a sensitive switch, active under physiological conditions, to control the state of aggregation of the virus proteins. Electrostatic interactions, particularly in the form of mutual repulsion by carboxylate groups, have been recognized for many years to be important in the assembly and disassembly of simple helical and spherical plant viruses (*2, 3*). TMV exhibits anomalous p*K* values near 7, and Caspar (*2*) suggested that these anomalous p*K*s might arise from the forced juxtaposition of carboxylate groups. These groups were predicted to be in subunit interfaces (*4*).

The three-dimensional structure of intact TMV has been determined by X-ray diffraction from oriented gels of the virus, using methods analogous to those of protein crystallography (*5, 6*). The structure was refined at 2.9 Å resolution to an *R* factor of 0.096 (*7*). The final model included all of the nonhydrogen atoms of both the RNA and the protein. The quaternary structure of the virion and the chain fold for two subunits of the virus are shown in Fig. 1. Three sites of electrostatic repulsion were identified: in the axial intersubunit interface, Glu50 from one subunit is close to Asp77 from a subunit above it; in the lateral interface, Glu95 from one subunit is close to Glu106 from the adjacent subunit; and in the RNA binding site, Asp116 is close to one of the RNA phosphates. The interaction between Glu50 and Asp77 is shown in Fig. 1B. It has been suggested (*7*) that all three charge pairs contribute to the anomalous titration behavior of TMV and that the Glu95/Glu106 pair and the Asp116/phosphate pair could be calcium binding sites. TMV has at least two sites for which calcium ions and protons compete (*8*).

The early stages of TMV infection could be described as follows (*7*). After the initial entry of a virion into a cell, the low calcium concentration and high pH (relative to the extracellular environment) remove protons and calcium ions from the carboxyl–carboxylate and carboxylate–phosphate pairs, allowing electrostatic repulsive forces from the negative charges to destabilize the virus. This destabilization is sufficient to expose the first start codon of the RNA. After destabilization, ribosomes can bind RNA in competition with the coat protein, completing the uncoating of the RNA in a process called cotranslational disassembly (*9*).

The hypothesis that specific residues are responsible for the electrostatic repulsion in disassembly can be tested by site-directed mutagenesis of the TMV coat protein followed by assays for disassembly of the intact virions. Examination of the virus structure suggests that changing Glu50 to Gln or Asp77 to Asn will affect only the electrostatic repulsion between the groups, having

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0042-6822/95 $6.00

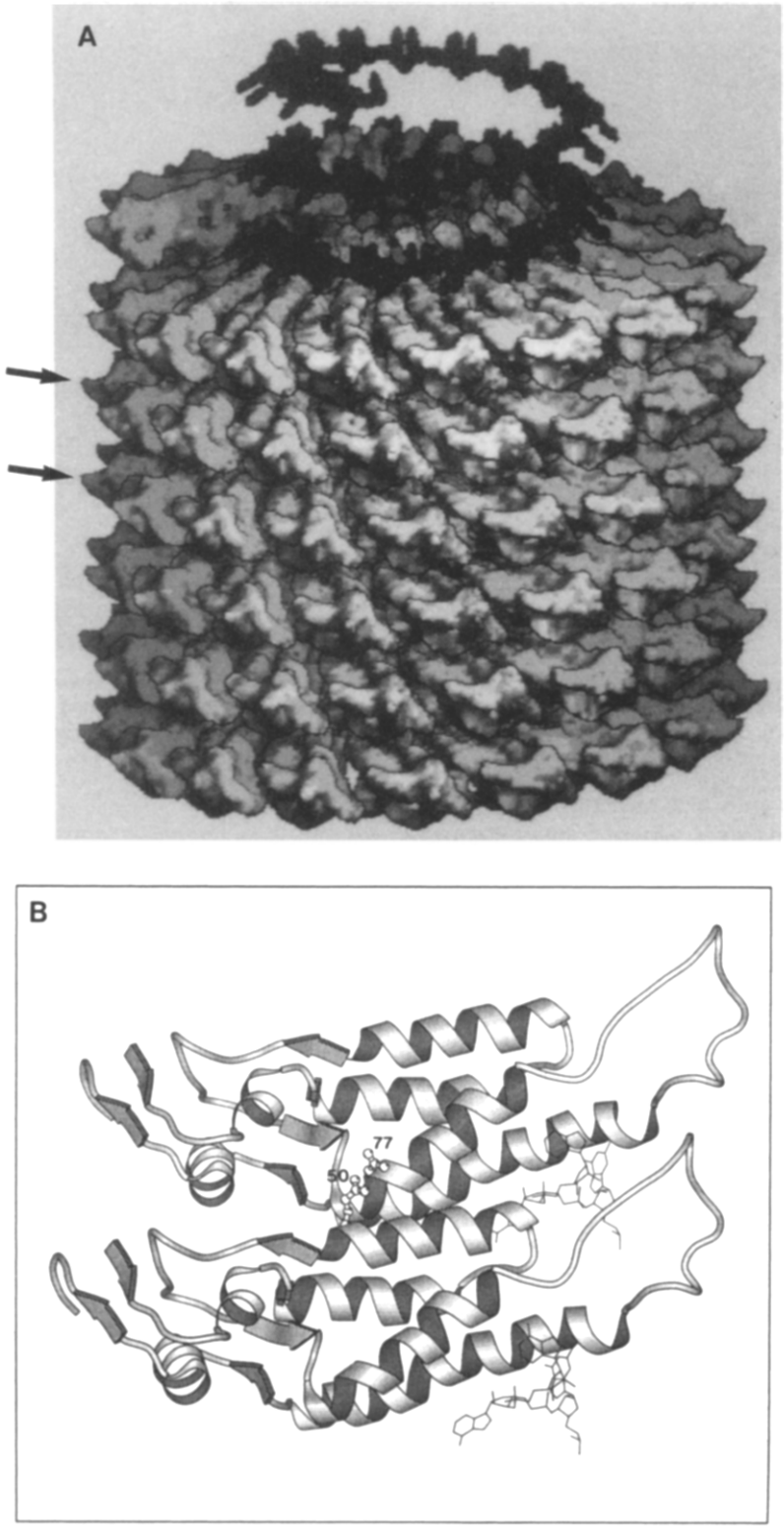

FIG. 1. Computer graphics representations of the tobacco mosaic virus structure. (A) A short segment (98 subunits) of the virus. Protein subunits are shown in light gray; RNA in dark gray. Two extra turns of the RNA chain are shown for clarity. The 3′ end of the RNA is at the top of the figure. (B) Ribbon drawing of two subunits of the coat protein. These subunits correspond to those shown by arrows in A. Glu50 from the bottom subunit and Asp77 from the top subunit are shown as detailed skeletal structures. This figure was generated by the program MOLSCRIPT (*30*).

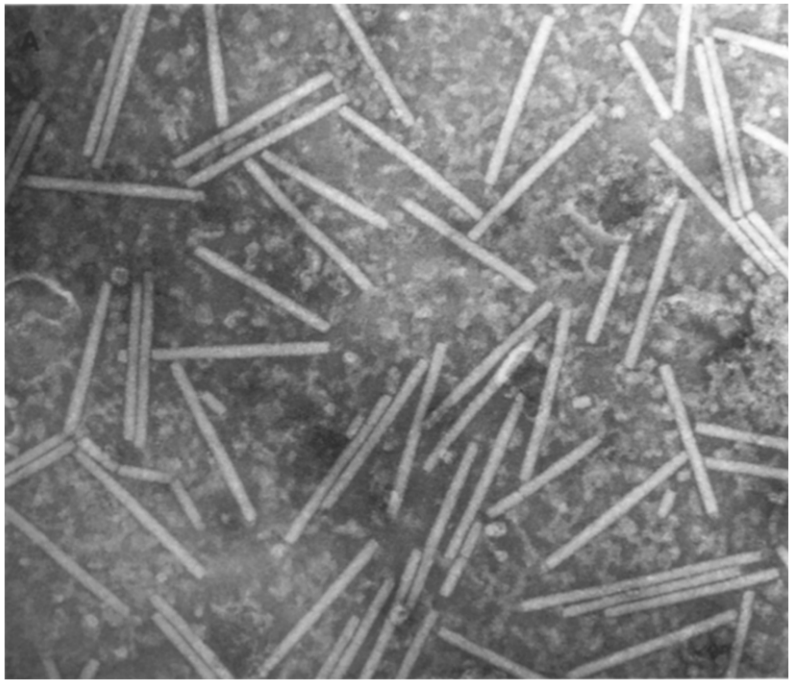

FIG. 2. Electron micrographs of leaf extracts from plants infected with (A) wild-type TMV, (B) TMV-E50Q, and (C) TMV-D77N. These extracts were used for the infectivity assays in Table 1. For electron microscopy, aliquots of 3 μl were spotted onto formvar-coated electron microscope grids and allowed to dry. Grids were then negatively stained with 1% uranyl acetate for 2 min and wicked dry with filter paper.

no effect on the surrounding hydrogen bonding patterns or hydrophobic interactions. We describe here the effects of these changes *in vivo* and *in vitro*. We refer to the viruses containing the mutations as TMV-E50Q and TMV-D77N, or simply as E50Q and D77N.

Site-directed mutagenesis (*10–12*) and *in vitro* transcriptions (*13, 14*) were performed as previously described. Transcription products were mechanically inoculated directly onto the leaves of *Nicotiana tabacum* L. cv. Xanthi. Ten days after infection, virions were purified as described (*15*), with a final centrifugation step in a 10–40% sucrose density gradient. Coat proteins were isolated by acetic acid degradation (*16*). TMV-E50Q and TMV-D77N were both able to infect tobacco (cv. Xanthi). Their capacity to move systemically within the plants was markedly reduced, however; the time from inoculation to the appearance of symptoms on upper noninoculated leaves was more than 2 weeks, compared with only 5–7 days for wild-type TMV. Systemic symptoms for E50Q and D77N also differed from the characteristic light-green dark-green mosaic produced by the wild-type virus, appearing instead as sporadic yellow patches.

Virions extracted from leaf tissue infected by either coat protein mutant appeared by electron microscopy to be morphologically normal (Fig. 2). Gel electrophoresis of RNA extracted from virions before density gradient purification showed, however, that the mutant virions contained very little RNA, compared with wild-type TMV. Even after density gradient purification, spectroscopic analysis showed that preparations of the E50Q virus-like particles contained only about 25% of the normal RNA complement (OD_{260}/OD_{280} = 0.9, compared with values close to 1.2 for wild-type TMV). Preparations of purified D77N particles contained about 70% of the normal RNA complement (OD_{260}/OD_{280} = 1.1).

An assay for viral disassembly was developed, based on the exposure of RNA to ribonuclease. Earlier workers (*17*) had obtained excellent qualitative results by measuring progressive changes in sedimentation behavior, electron microscopic appearance, and electrophoretic mobility and were able to show that the process of disassembly of TMV includes a number of stable intermediate particles. These assays were not quantitative, however, because any quantitative assay based on particle size would require a potentially unstable mathematical model that took into account the lengths and populations of all possible intermediate particles. Such an assay would also be affected adversely by errors in the measurement of the contributions of extensively or completely degraded particles. Results from sedimentation and electron microscopic assays would be further obscured by the presence of helical protein aggregates that did not contain RNA; as noted above, such aggregates are significant contaminants of our mutant virion preparations. In order to avoid these problems, a simple, quantitative assay was developed in which the amount of viral nucleic acid freed from the disassembling virus particles was measured directly.

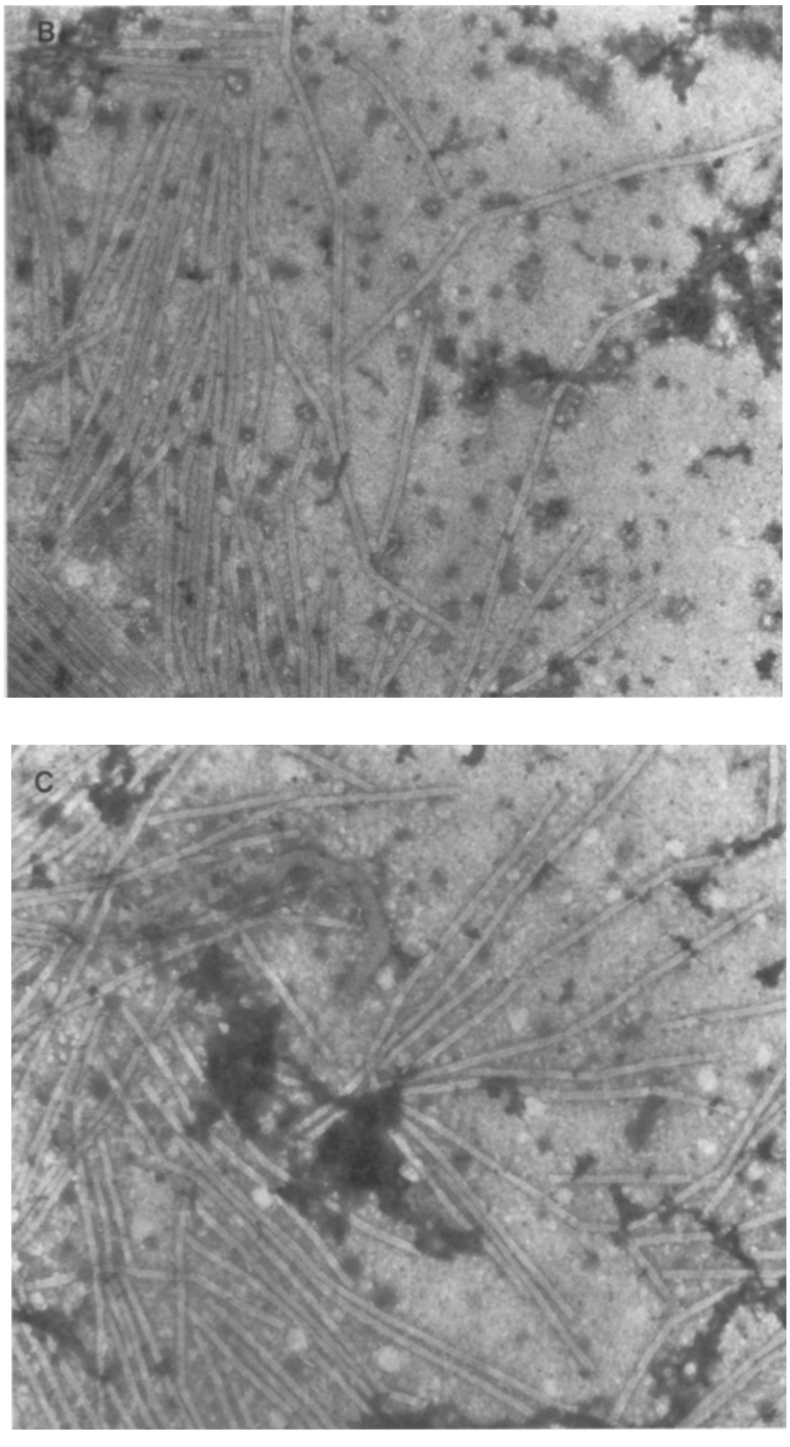

FIG. 2—*Continued*

For disassembly assays, samples were dialyzed against at least 200 volumes of water overnight at about 5°, and concentrations were determined by absorption at 260 nm corrected for light scattering, using an extinction coefficient of 3.01 $mg^{-1}cm^2$ (*18*). The samples were adjusted to a concentration of 2.0 mg/ml, carbonate–bicarbonate buffer (pH 10.5) was added to 0.02 *M*, and the solution was kept on ice, removing aliquots at intervals as required. Aliquots at zero incubation time were taken before addition of carbonate–bicarbonate buffer.

The aliquots were adjusted to pH 7.0 ± 0.5 with 0.2 *M* HCl and made 0.1 *M* in KCl. Bovine pancreatic ribonuclease was added to about 3 μg/ml, and digestion was allowed to proceed at room temperature for 1 hr. The

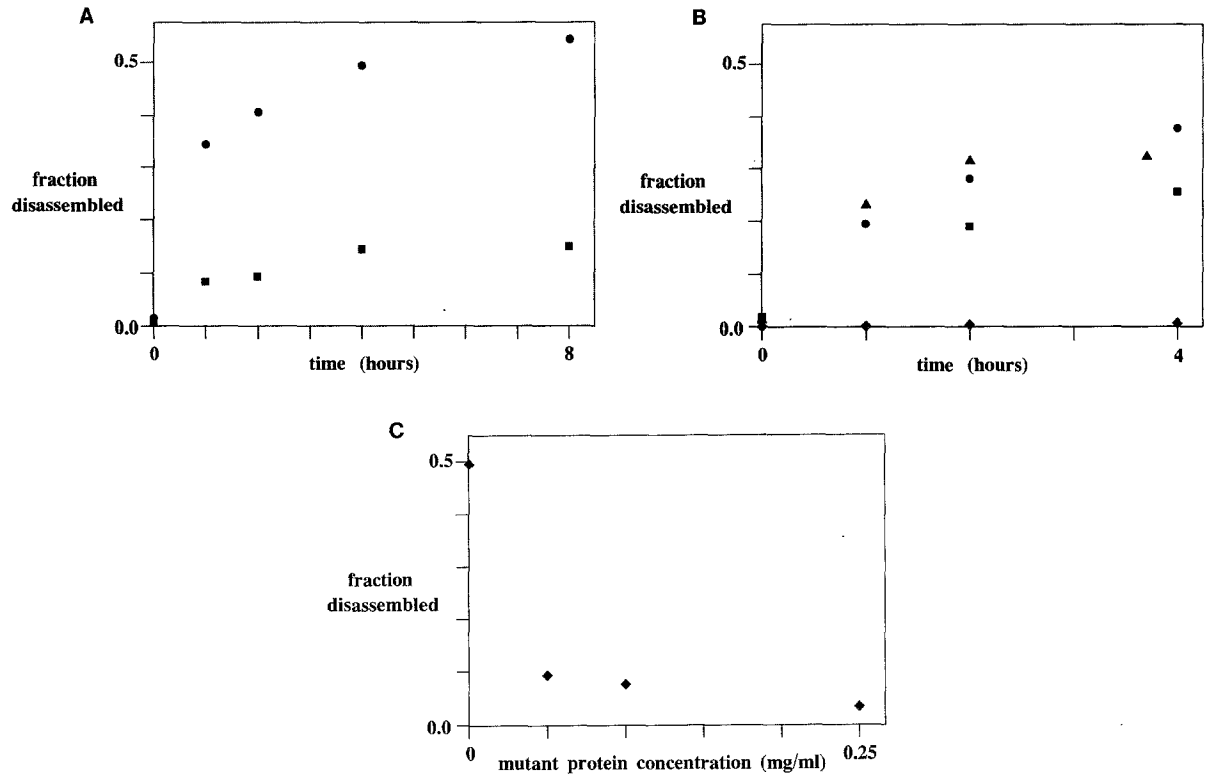

FIG. 3. Alkaline disassembly of TMV mutants. The fraction of virus disassembled was determined by measuring the concentration of free nucleotides produced, as described in the text. (A) Disassembly of TMV and TMV-D77N at high pH over an 8-hr period. ●, wild-type TMV; ■, TMV-D77N. (B) Disassembly of wild-type TMV at high pH in the presence of added coat protein. ●, no coat protein added; ▲, wild-type TMV coat protein added; ■, D77N coat protein added; ◆, E50Q coat protein added. (C) Disassembly of wild-type TMV as a function of concentration of E50Q protein. Virus was incubated at high pH in the presence of E50Q protein for 4 hr. The fraction of wild-type TMV with no protein added measured as disassembled at the beginning of this experiment was 0.016.

pH was then reduced to 3.4 ± 0.4, sufficient to observe turbidity due to aggregated protein and partially degraded virus. The samples were centrifuged for 3 hr at 400,000 *g* to remove protein and undegraded virus, then brought to pH 7.0 ± 0.5 with 0.2 *M* sodium carbonate. Absorption at 260 nm was used to determine free nucleotide concentrations, assuming an extinction coefficient for the mixture of nucleotides of $1.0 \times 10^4\ M^{-1}cm^{-1}$.

Alkaline disassembly of D77N and wild-type TMV was followed over a period of 8 hr. The fractions disassembled at various times during the incubation are given in Fig. 3A. D77N was significantly more stable than wild-type TMV, exposing only about one-third the number of nucleotides in any given time. Alkaline disassembly of E50Q was not measured because of the small amounts of RNA in the particles.

Alkaline disassembly of wild-type TMV in the presence of excess wild-type or mutant coat protein was also measured. These experiments were carried out as described above, except that all solutions were 0.1 *M* in KCl, since overnight dialysis of TMV coat protein against water tended to cause denaturation of the protein. The course of disassembly of TMV in the presence of free coat protein (virus to free protein ratio 8:1) is shown for wild-type, D77N, and E50Q protein in Fig. 3B. Disassembly of TMV under these conditions was not significantly affected by the presence of excess wild-type coat protein, but it appeared to be reduced by D77N protein, and was virtually abolished by E50Q protein. The effect of E50Q protein concentration on disassembly of wild-type virions is shown in Fig. 3C.

Infectivity assays were performed by grinding 0.1 g of infected Xanthi tissue in 300 μl of 0.01 *M* phosphate buffer, pH 6.8, at 0°. A Carborundum-dusted half-leaf of the local lesion host *N. tabacum* cv. Xanthi-nc was inoculated mechanically with 50 μl of the plant extract; the other half-leaf was inoculated with wild-type TMV-infected plant extract as a control. Infectivity of purified virions was determined by inoculating each half-leaf with 50 μl of a solution containing 0.02 mg/ml virions. Infectivity was measured as the number of lesions produced on the half-leaf.

Infectivity from equivalent amounts of infected plant sap was markedly lower for E50Q and D77N than for wild-type TMV (Table 1). The infectivity of purified D77N virions was also found to be significantly less than that of

TABLE 1

INFECTIVITY ASSAYS

Inoculum	Number of leaves	Mean lesions/ half-leaf	Control	Mean lesions/ half-leaf
TMV-E50Q (plant extract)	3	15.0 ± 2.2 (9.2)	TMV (plant extract)	≫300
TMV-D77N (plant extract)	3	6.7 ± 1.5 (5.4)	TMV (plant extract)	≫300
TMV-D77N (purified virions)	8	140.8 ± 4.2 (57.1)	TMV (purified virions)	366.9 ± 6.8 (156.8)
TMV (pH 9)	5	168.4 ± 5.8 (157.0)	TMV (pH 7)	350.2 ± 8.4 (108.5)
TMV-D77N (pH 9)	6	149.0 ± 5.0 (105.0)	TMV-D77N (pH 7)	117.3 ± 4.4 (69.6)
TMV with E50Q protein	8	5.3 ± 0.8 (3.2)	TMV with wild-type protein	111.8 ± 3.7 (81.3)
TMV with D77N protein	8	32.9 ± 2.0 (10.3)	TMV with wild-type protein	34.3 ± 2.1 (12.9)

Note. One-half of each leaf was inoculated with mutant TMV-infected plant extract or purified virions and the other half with an equivalent amount of the specified control. Numbers of local lesions given as ≫300 could not be accurately counted because lesions were confluent throughout the half-leaf. Mean numbers of lesions are given ± a standard deviation calculated as $\sqrt{N/n}$, where n is the number of leaves observed, and N is the total number of lesions on all n half-leaves. This standard deviation calculation is based on the assumption that the number of lesions conforms to a Poisson distribution and that the relevant observation is the total number of lesions counted. The much larger standard deviation given in parentheses is the more conventional standard deviation, calculated from the variation among half-leaves. This standard deviation is not, however, appropriate for a half-leaf assay; it measures primarily scatter among leaves, which is irrelevant to a half-leaf assay.

wild-type virions. In contrast to wild-type TMV, however, purified D77N virions maintained their levels of infectivity when the pH of the inoculum was raised from 7.0 to 9.0 (Table 1). Virion infectivity experiments were not performed for E50Q because of the small amounts of RNA in the particles.

In view of the ability of the mutant coat proteins to suppress wild-type TMV disassembly, their effect on the infectivity of wild-type TMV was also measured. Incubation of TMV virions at pH 8.0 is conducive to ribosome binding, and it has been suggested that the process involves the removal of a small number of coat protein subunits from the 5′ end of the RNA (*9*). We therefore reasoned that exposure of wild-type virions to pH 8.0 in the presence of excess mutant coat protein should allow exchange of wild-type for mutant protein subunits. Wild-type virions (0.4 μg) were incubated with 4.0 μg of purified coat protein for 15 min in 0.01 *M* Tris–HCl, pH 8.0. The pH was then adjusted with an equal volume of 0.1 *M* Tris–HCl, pH 7.4, and the sample incubated for an additional 30 min. Infectivity was determined as above, with half of each leaf receiving virions treated with excess wild-type coat protein, while the other half received virions treated with either excess D77N or E50Q coat protein. Incubation with excess D77N coat protein did not have any significant effect on the infectivity of wild-type virions, but the infectivity of wild-type virions incubated with excess E50Q coat protein was reduced by about 95% (Table 1).

The observations reported here confirm the hypothesis that the electrostatic repulsion between Glu50 and Asp77 contributes significantly to the efficient disassembly of TMV. Replacement of the members of carboxyl–carboxylate pairs in the coat protein subunit interface by the corresponding amides clearly stabilized protein–protein interactions. In the case of D77N, the stability of the virions was significantly enhanced (Fig. 3A). Under some conditions, free TMV coat protein may inhibit viral disassembly (*19*), but under the conditions used here we observed no such effect (Fig. 3B). Excess protein in the D77N preparation was therefore not in itself responsible for the slow disassembly of D77N. It is not possible to determine from our data whether the rate of disassembly was reduced at all stages or whether the stability of some partially disassembled intermediate (*17*) was so greatly increased that no further disassembly took place. Regardless, these data provide direct evidence that electrostatic repulsion between juxtaposed carboxylate groups drives viral disassembly.

E50Q did not form virions in sufficient yield to assay virion stability. It did, however, form filamentous assemblies in the plant that were morphologically extremely similar to intact virions, but contained little or no RNA. Wild-type TMV protein forms helical aggregates at pH values below about 6.5 (*20*); these aggregates are structurally isomorphous with the intact virus (*21, 22*). It appears that in E50Q, the removal of the repulsive forces between Glu50 and Asp77 stabilizes the helical protein aggregates to the point where they become the predominant assembly under plant physiological conditions. The failure to form intact virions follows from the stability of the helical aggregates; virion formation requires the presence of smaller, 20S protein aggregates (*23*), which predominate in the wild-type virus protein at neutral pH (*24*). The formation of protein helical aggregates thus provides further, albeit indirect, evidence for the impor-

tance of the repulsion between Glu50 and Asp77 in virion stability.

The reduced infectivity of D77N and E50Q (Table 1) is consistent with their forming unusually stable virions; the reduction is not due to the reduced formation of intact virions alone, as is shown by the reduced infectivity of purified D77N virions relative to wild-type virions. The ability of D77N to retain its infectivity at pH 9, when the infectivity of wild-type TMV is significantly reduced (Table 1), is consistent with the greater resistance of D77N to alkaline degradation. Long-distance transport of tobamoviruses within their hosts is correlated with the presence of intact virions (*25*), which probably disassemble at the distal site of infection; the reduction in efficiency of long-distance transport of the mutant viruses is again consistent with unusual stability of the virions.

The ability of the mutant coat proteins to suppress disassembly of wild-type TMV (Figs. 3B and 3C) demonstrates that disassembly is at least partially a reversible process. Subunits of mutant protein can bind to a partially disassembled virion, forming a complex much more stable than the wild-type partially disassembled virion, and thus effectively inhibiting further disassembly. The different degrees of inhibition exhibited by D77N and E50Q are a consequence of the structure of the virus and the polar nature of disassembly. Disassembly of TMV virions begins at the 5′ end of the RNA (*26*); the exposed disassembling face contains Asp77, but not Glu50 (Fig. 1). An incoming subunit of E50Q will bind very strongly to the exposed face, forming an intersubunit hydrogen bond between Gln50 and Asp77, but making no repulsive electrostatic interaction. The complex will thus be much stabler than the complex of wild-type subunits. An incoming subunit of D77N, in contrast, will make wild-type interactions with the exposed face, including the normal repulsive interaction between Asp77 (from the wild-type protein) and Glu50 (from the mutant protein). D77N may have a stabilizing effect due to the binding of a wild-type or D77N protein subunit to the already bound D77N subunit, but this effect will be second-order and much smaller than the direct effect seen in E50Q. The ability of the mutant coat protein E50Q to reduce the infectivity of wild-type TMV (Table 1) is consistent with its ability to suppress wild-type TMV disassembly, and probably involves the exchange of mutant and wild-type protein subunits under the partially disassembling conditions of incubation.

Transgenic plants expressing the coat proteins of many viruses, including TMV, resist infection by the corresponding virus (*27, 28*). One possible mechanism for this type of resistance is the inhibition of disassembly by the coat protein molecules (*19, 29*). Our observations suggest that mutations of the type that we have made could be used to investigate this mechanism, and could possibly increase the resistance of transgenic plants to infection.

ACKNOWLEDGMENTS

This work was supported by NSF Grant MCB 9207970 and USDA Grant 90-37262-5384. We thank Dr. Hong Wang for valuable discussions and assistance with figures.

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